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TECHNICAL ADVANCE

Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene

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Summary

The inability of many higher eukaryotes to convert 5-fluorocytosine to cytotoxic 5-fluorouracil presents the possibility of using the bacterial cytosine deaminase *codA* gene for negative selection. In transformed plant callus, expression of *codA* results in cell death on 5-fluorocytosine. In transgenic tobacco and *Lotus japonicus* plants the substrate-dependent negative marker segregates as a single dominant gene, and on 5-fluorocytosine CodA$^+$ seedlings stop growing at the early seedling stage. Positive selection of CodA$^+$ tobacco on the pyrimidine biosynthetic inhibitor *N*-(phosphonacetyl)-L-aspartate was obtained, by pyrimidine salvage from external cytosine. Activity of cytosine deaminase was determined by conversion of labelled cytosine to uracil followed by separation in thin layer chromatography. The *codA* marker therefore provides substrate-dependent negative and positive selection, together with cytosine deaminase reporter activity.

Introduction

The use of positive genetic markers conferring resistance towards antibiotics or herbicides is crucial for the selection of transformed plant cells and a prerequisite for the production of transgenic plants. To establish refined cellular and genetic techniques, as for example gene targeting by homologous recombination, negative marker genes counter-selecting cells expressing the gene are also essential. Developmentally programmed cell death following expression of a cell autonomous negative marker could provide new possibilities for the analysis of cell lineages and cell differentiation processes. At present two negative plant markers have been described. The *Agrobacterium tms2* gene encoding indole-3-acetamide hydrolase converts supplied indole-3-acetamide substrate into toxic levels of the auxin indole-3-acetic acid (Depicker *et al.*, 1988). Recently, a constitutively expressed nitrate reductase gene was used as a negative selection marker on nitrate-deficient growth medium. In tobacco the endogenous nitrate reductase was not induced under these conditions and negative selection was possible on chlorate (Nussaume *et al.*, 1991). A novel substrate-dependent negative selection marker based on the *Escherichia coli* cytosine deaminase *codA* gene is presented here. When *E. coli* utilizes cytosine through the pyrimidine salvage pathway, cytosine permease and cytosine deaminase mediate the uptake and conversion of cytosine into uracil (Danielsen *et al.*, 1992) (Figure 1). Cytosine deaminase also deaminates the innocuous 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), a precursor of 5-fluoro-dUMP which irreversibly inhibits thymidylate synthase activity. Consequently the cells are deprived of dTTP for DNA synthesis (Brockman and Anderson, 1963). In plants and mammals enzymatic conversion of cytosine to uracil is absent and cytosine appears biochemically inert (Bendich *et al.*, 1949; Ross, 1965). In mammals, low toxicity of administered 5-FC is reflected in rapid and almost quantitative excretion (Koechlin *et al.*, 1966).

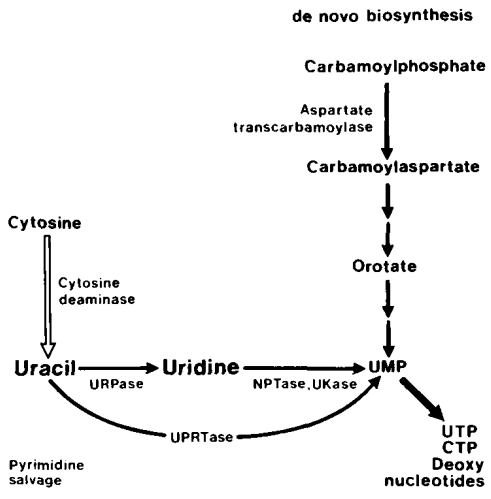

Figure 1. Schematic outline of the plant pathway for UMP biosynthesis, and pyrimidine salvage (Kanamori *et al.*, 1980).
The first committed step of *de novo* synthesis mediated by aspartate transcarbamoylase is at the top; pyrimidine salvage from uracil is left to right. Cytosine deaminase mediated conversion of cytosine to uracil in *codA* transgenic plants is indicated by an open arrow. URPase, uridine phosphorylase; UKase, uridine kinase; NPTase, nucleoside phosphotransferase; UPRTase, uracil phosphoribosyltransferase.

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Negative selection of mammalian culture cells on 5-FC was, however, observed after transfection with a marker gene expressing bacterial cytosine deaminase (Mullen *et al.*, 1992). This paper describes the use of cytosine deaminase as a dual selection marker and reporter gene in transgenic plants. It is shown that 5-FC does not affect the growth of wild-type plants, while transgenic plants expressing cytosine deaminase are negatively selected on 5-FC. The biochemical mechanisms behind the dual selections are discussed.

Results

Construction of the selectable marker

The cytosine deaminase coding sequence introduced into tobacco and the diploid legume *Lotus japonicus* was obtained from the plasmid pSD112 carrying the *codBA* operon (Danielsen *et al.*, 1992). Using PCR techniques the *E. coli* GTG translational start codon was changed to ATG before *codA* was fused to the nopaline synthase 3′ region and either the cauliflower mosaic virus 35S promoter (pNE7) or the 2′ gene promoter (pNE5) (Figure 2). Transgenic plants of *L. japonicus* (pNE7) and tobacco (pNE5) were obtained using described *Agrobacterium* procedures (Handberg and Stougaard, 1992; Horsch *et al.*, 1985). To confirm the presence of intact cytosine deaminase constructs in transgenic *Lotus* and tobacco plants, and to probe for cross-hybridization, genomic DNA was digested with *Cla*I (Figure 2) and hybridized to the *codA* coding sequence (Figure 3a). Transcription of the *codA* marker gene was demonstrated by hybridization of total RNA from leaves with the *codA* coding sequence. Transcript hybridization signals corresponding to the expected approximately 1400 nucleotides were detected in *codA* plants (Figure 3b). No cross-hybridizing RNA species were detected in *Lotus* and tobacco control plants, demonstrating the potential use of *codA* as a reporter gene for measuring transcript levels.

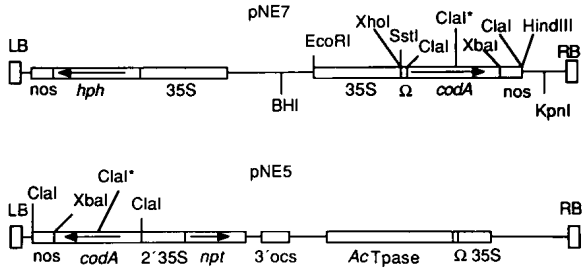

Figure 2. Schematic representation of the *codA* gene constructs used for expression of the bacterial cytosine deaminase in plants.
In the pNE7 plasmid, the 35S*codA* 3′*nos* gene was cloned into the binary vector pLX412 (Landsmann *et al.*, 1988). The 2′*codA* 3′*nos* gene of pNE5 was constructed by inserting a *codA* 3′*nos* *Cla*I fragment downstream of the 2′ promoter of the pSLJ1111 plasmid (Scofield *et al.*, 1992), replacing the GUS 3′*nos* fragment. **Cla*I site Dam methylated. Not to scale.

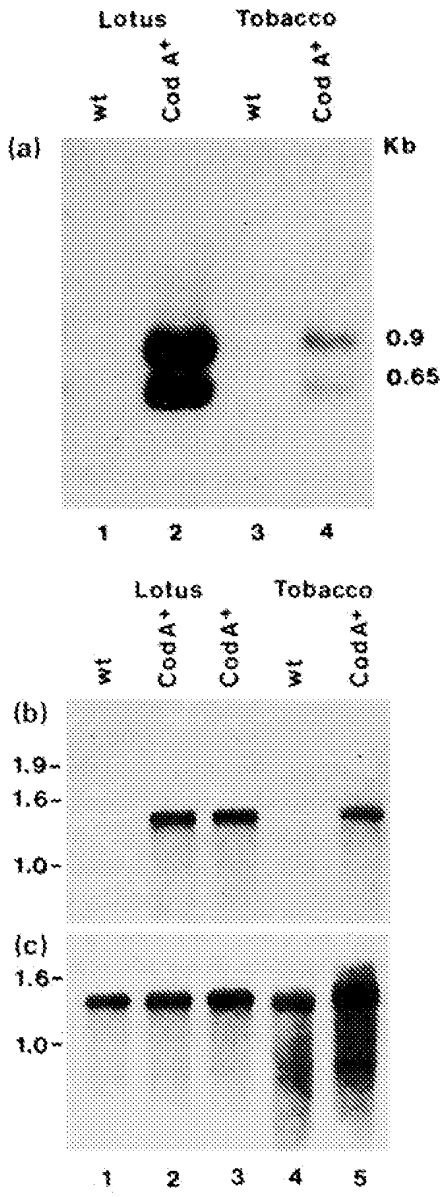

Figure 3. Southern and Northern analysis of transgenic *codA* plants.
(a) DNA extracted from *Lotus japonicus* and tobacco plants digested with *Cla*I. The 0.9 kb and 0.65 kb fragments encompassing the *codA* coding sequence and the 3′*nos* region (Figure 2), are detected in the transgenic plants (lanes 2 and 4) after hybridization with the *codA* coding region. No cross-hybridization in control plants (lanes 1 and 3). (b) Three micrograms of total RNA from *Lotus* and 12 μg total RNA from tobacco plants were hybridized with the *codA* coding sequence, or, (c) a cDNA probe for the constitutively expressed ubiquitin genes (Wiborg *et al.*, 1985). Transcripts of approximately 1400 nucleotides are detected by the *codA* probe in the transgenic *Lotus* and tobacco plants (lanes 2,3 and 5). Control plants represented by lanes 1 and 4.

Negative selection in calli

In callus medium the cytotoxic 5-FU prevented growth and caused death of *L. japonicus* calli, at levels of 50–100 μg ml^{-1} (Figure 4c). Concentrations of 5-FC up to 2 mg ml^{-1} had no observable effects. The negative selection was therefore tested under tissue culture conditions by

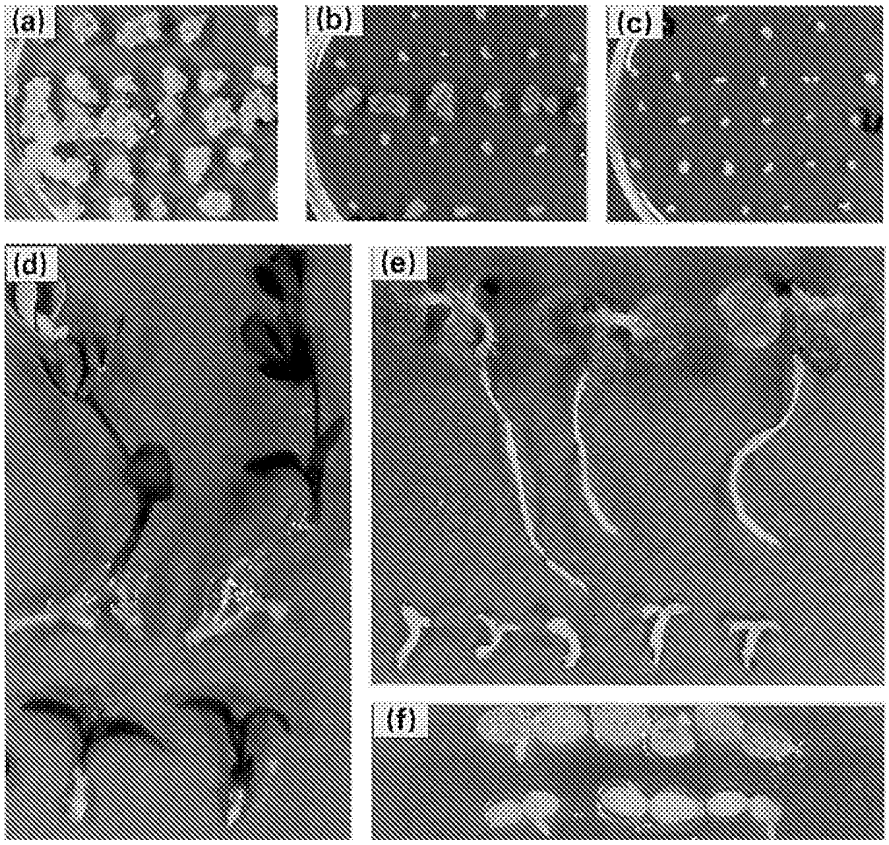

Figure 4. Negative and positive selection using 5-fluorocytosine and *N*-(phosphonacetyl)-L-aspartate.
Slices of *Lotus japonicus* calli carrying 35S*codA* 3′*nos* (two top and two bottom rows) and calli transformed with the pLX412 vector (middle row), were grown for 2 weeks. (a) Callus medium. (b) Callus medium containing 1 mg ml^{-1} of 5-fluorocytosine. (c) Cytotoxic effect of 100 μg ml^{-1} 5-fluorouracil. (d,e) Phenotypes of *Lotus japonicus* and tobacco F_1 plants germinated and grown on 250 μg ml^{-1} of 5-FC, $CodA^0$ (top) and $CodA^+$ (bottom). (f) Phenotypes of $CodA^+$ (top) and $CodA^0$ (bottom) F_1 tobacco plants germinated on 400 μg ml^{-1} PALA plus 50 μg ml^{-1} cytosine.

moving *codA* transformed *Lotus* calli from callus medium (Handberg and Stougaard, 1992) to similar plates containing 5-FC at 500 μg ml^{-1} or 1 mg ml^{-1} (Figure 4b). To increase 5-FC uptake small pieces of calli were transferred. Transgenic calli carrying the 35S*codA* 3′*nos* marker gene did not grow and were white in appearance. Control calli grew normally and were green. In an attempt to demonstrate that the effect of 5-FC depends directly on the cytosine deaminase activity, concentrations of cytosine up to 2 mg ml^{-1} and uracil up to 100 μg ml^{-1} were added to plates containing 5-FC at 500 μg ml^{-1}. Unexpectedly, neither of the two pyrimidines counteracted the negative selection and uracil at concentrations higher than 50 μg ml^{-1} even enhanced the effect of 5-FC. Differences in uptake, enzyme affinities, or metabolic control effects may explain these observations.

Negative selection in plants

To test the negative selection *in planta*, transgenic *Lotus* and tobacco plants were selfed and the F_1 seeds germinated on 250 μg ml^{-1} 5-FC. Generally, germination of $CodA^+$ seeds was slow, root growth was inhibited and roots did not elongate into the agar. Shoot development was rudimentary and in *Lotus* only a single trifoliate leaf appeared (Figure 4d). Tobacco seedlings were small and white (Figure 4e). Growth of control plants or segregating transgenic $CodA^0$ plants were not observably affected by 5-FC. The viability of $CodA^+$ *Lotus* and tobacco plants and the possible rescue after negative selection were tested by transfer to non-selective plates containing orotate at 100 μg ml^{-1}. Resumption of growth occurred in around 40% of the plants of both species.

The stability of the negative selection marker was investigated by following the segregation of *codA* in the F_1 generation of four independent *Lotus* plants and three independent tobacco plants. Cytosine deaminase activity was determined both by direct biochemical assay in non-selected plants, and by the ratio of sensitive to unaffected plants on 5-FC plates (Table 1a). Irrespective of the method used, *codA* segregated in the 3:1 ratio expected for a single dominant marker. A minor deviation between the two ratios observed in some F_1 populations, e.g. the *Lotus* line of Table 1a, could be statistical variation or could arise from intrinsically poorly growing $CodA^0$ seedlings scored as $CodA^+$ on selective medium. Indeed, three seedlings without cytosine deaminase activity were found among 125 *Lotus* and tobacco seedlings scored as $CodA^+$. The negative selection appears quite tight as no cytosine deaminase activity was found in 208 *Lotus* and tobacco plants originally assigned the $CodA^0$ character. Lack of penetrance has so far not been detected.

Table 1. (a) Segregation of cytosine deaminase activity and 5-fluorocytosine sensitivity in the F_1 plants from one line of selfed *L. japonicus* (35S*codA*) and one line of selfed tobacco (2′*codA*). Plants for biochemical assay were grown without selection. (b) Segregation of PALA resistance and cytosine deaminase activity in F_1 plants from selfed tobacco transformed with pNE5. Cytosine deaminase activity was determined in plants rescued from selective plates

				Assay, $CodA^+$:$CodA^0$		
	No. of plants	Ratio	χ^2	No. of plants	Ratio	χ^2
(a) Growth test on 5-FC, sensitive: unaffected						
Lotus	105:27	3.9:1	1.45	57:21	2.7:1	0.15
Tobacco	165:58	2.9:1	0.13	55:20	2.8:1	0.11
(b) Growth test on PALA+cytosine, sensitive:resistant						
Tobacco	46:118	1:2.6	0.81	121:43	2.8:1	0.13

The predicted counteractive dilution effect of other pyrimidines was tested on tobacco. Seeds segregating *codA* were germinated on plates containing 150 μg ml^{-1} 5-FC and a molar excess of the following compounds: *cytosine, uracil, thymine, cytidine, uridine, thymidine,* cytosine and thymidine, uracil and thymidine. At the concentrations used, these compounds failed to reverse the negative effect of 5-FC; uracil again enhanced the effect. A combination of thymidine, deoxycytidine, and cytidine was more effective and reverted the shoot phenotype of $CodA^+$ tobacco plants to almost wild-type appearance. Apparently, other fluorinated pyrimidine compound(s), in addition to 5-fluoro-dUMP, interfere with pyrimidine metabolism.

Positive selection in tobacco

The potential use of cytosine deaminase as a dual purpose marker also providing substrate-dependent positive selection was investigated in tobacco grown on *N*-(phosphonacetyl)-L-aspartate (PALA), an inhibitor of the pyrimidine biosynthetic enzyme aspartate transcarbamoylase (Swyryd *et al.*, 1974; Figure 1). Growth and development of wild-type tobacco were retarded by 400 μg ml^{-1} PALA and positive selection for $CodA^+$ seedlings surviving on cytosine uptake through the pyrimidine salvage pathway, appeared feasible. On PALA cotyledons were yellow, and no roots developed. Addition of 50 μg ml^{-1} of cytosine to selective plates resulted in green cotyledons on transgenic $CodA^+$ tobacco seedlings, but the growth inhibition was not reversed (Figure 4f). The phenotypic difference between $CodA^+$ and $CodA^0$ seedlings was therefore not as clear as after negative selection, and the relationship between green cotyledons and cytosine deaminase activity was determined. Table 1b shows the 3:1 ratio between green and yellow cotyledons and compares directly with the cytosine deaminase activity determined in the same plants, rescued on medium containing orotate at 100 μg ml^{-1}. The enzymatic assays confirm the scored phenotypes with an error of less than 10%. Uridine or orotate reversed the effect of PALA in any genotype whereas uracil reversed the effect on wild-type tobacco to the same extent as cytosine in $CodA^+$ plants. Slow conversion of uracil into uridine or UMP could explain these differences.

The cytosine deaminase assay

A marker gene with an easy assay is useful in genetic segregation studies and allows direct correlation of gene expression with phenotype. For plant purposes, a modified (Andersen *et al.*, 1989) cytosine deaminase assay was therefore established. Crude extracts prepared from leaves homogenized with a 10-fold excess of extraction buffer were incubated with labelled cytosine. The uracil formed was subsequently separated from the substrate on cellulose TLC plates (Figure 5). Two substrate concentrations higher than the K_m of 0.53 mM (Andersen, 1979) were tested: (i) 0.9 mM 2-^{14}C cytosine; (ii) 0.9 mM 2-^{14}C cytosine plus 10 mM cytosine. For both reactions a linear relation between protein content and activity was observed up to a protein concentration of 2.1 μg μl^{-1} (crude 1:1). The detection limits for the assays were found at protein concentrations of 0.0042 μg μl^{-1} and 0.042 μg μl^{-1}, respectively (Figure 6a). For detection of low level activities the most sensitive condition was chosen as standard assay. To test the validity of this assay, activity was measured as a function of time (Figure 6b and c). Activity was linear for 1 h and the specific activity was 0.95 nmol uracil μg^{-1} protein h^{-1}. Approximately 15% activity was lost in 3 h. Therefore, the cytosine deaminase enzyme appears to be stable, allowing the employment of *codA* as a reporter gene for studies of gene expression in transgenic plants.

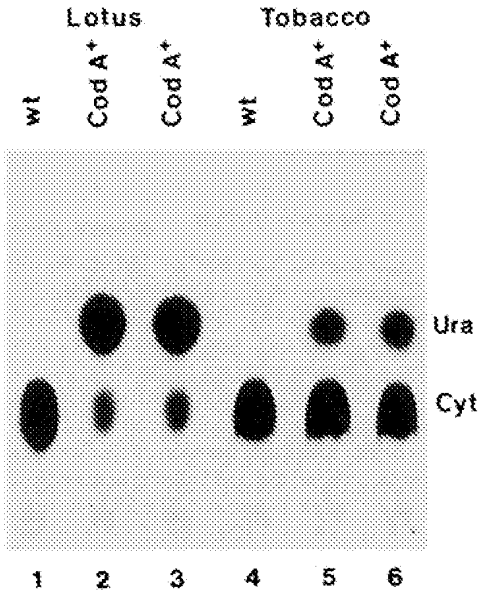

Figure 5. Determination of cytosine deaminase activity in plants. Conversion of the 2-^{14}C-cytosine (Cyt) substrate to 2-^{14}C-uracil (Ura) in extracts of CodA$^+$ *L. japonicus* and tobacco plants, lanes 2, 3, 5 and 6. Control plants, lanes 1 and 4.

Discussion

Expressed from the 35S promoter or the weaker 2′ promoter, cytosine deaminase provides a convenient negative selection in both *L. japonicus* and tobacco by converting the innocuous 5-FC to the cytotoxic 5-FU. The cytosine deaminase activity in the presence of 5-FC results in a distinct phenotype in both *in vitro* grown plant cells and plants. Judging by the absence of shoot development and root elongation on the sensitive plants, cell division in the root and shoot meristems appears to be inhibited or abolished. Rescue of around 40% of the growth-inhibited CodA$^+$ plants from 5-FC plates was nevertheless possible with both *Lotus* and tobacco. It will be interesting to test whether the negative effect is cell autonomous and *codA* therefore usable in programmed cell death experiments. This application will rely on 5-FC translocation throughout the plant and requires that fluorinated pyrimidines like 5-FU are not exported. The effect on the shoot meristem indicates good translocation, but the experiments reported can not exclude effects from exported fluorinated pyrimidines.

Some questions concerning the exact mechanism of the negative selection in plants are still unanswered. The inability of thymidine to reverse the 5-FC effect and the only partial reversion of the negative selection in tobacco observed with a combination of thymidine, deoxycytidine, and cytidine suggest that secondary effects of fluorinated pyrimidines might contribute to the negative selection in addition to inhibition of DNA synthesis. Inhibition of RNA synthesis and the appearance of a number of fluorinated pyrimidine compounds after administration of 5-FU was previously described for mammals (Harbers *et al.*, 1959). Support for the primary 5-fluoro-dUMP inhibition of plant thymidylate synthase activity came from growth of tobacco on the precursor 5-fluoroorotate (5-FOA). The strong

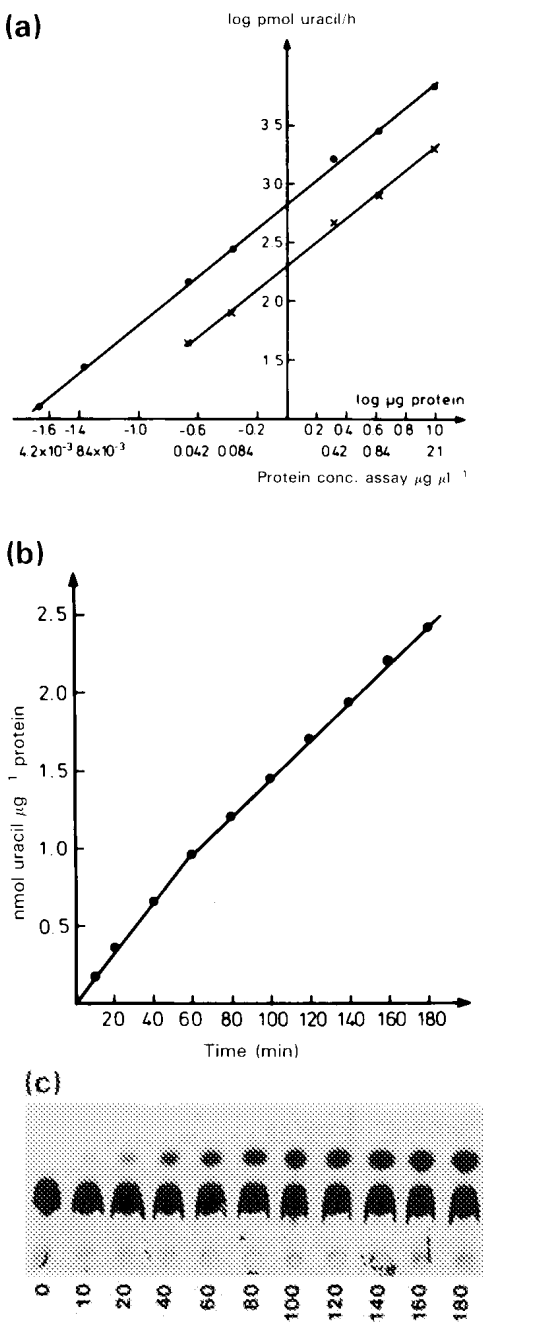

Figure 6. Cytosine deaminase activity from the 35S*codA* 3′*nos* reporter gene in *L. japonicus*.
(a) Double logarithmic plot of cytosine deaminase activity as a function of protein content. (•) Standard assay. (×) Assay with 10 mM cytosine. The actual protein concentrations in µg µl^{-1} are also given. (b) Cytosine deaminase activity measured by the standard assay as a function of time. (c) Autoradiogram of a time course experiment used for (b), protein concentration 0.06 µg ml^{-1}.

inhibitory effect of 5-FOA on shoot growth was reversed by thymidine alone (data not shown). To explain the above observations, the ineffective cytosine reversion, and the uracil enhancement of the 5-FC effect, additional knowledge of pyrimidine uptake, translocation, enzyme affinities and metabolic control mechanisms of the pyrimidine pathways is needed.

The positive selection is based on simultaneous inhibition of *de novo* synthesis and provision of an alternative biosynthetic route. Unfortunately the conversion of cytosine into UMP appears to be to slow in counteracting the effect of PALA completely. Results from pea (Bressan *et al.*, 1978) and *Vinca* (Kanamori *et al.*, 1980) suggest that conversion of uracil into uridine or UMP could be the rate-limiting step. Constitutive expression of a gene encoding uracil phosphoribosyltransferase together with the cytosine deaminase might therefore improve the positive selection, although pyrimidine uptake may also be limiting. Some additional investigation of alternative inhibitors and the selective conditions used might also provide more distinct phenotypes. Improvements are clearly necessary for species with larger seeds like *Lotus*, where positive selection under conditions used for tobacco is not possible.

The simple enzymatic assay and the independence of growth conditions used for plant cell culture or plant growth, makes the *codA* selection an attractive alternative to the negative selections based on indole-3-acetamide hydrolase (Depicker *et al.*, 1988) or nitrate reductase (Nussaume *et al.*, 1991). Certainly, lack of cytosine deaminase activity in barley, sugar beet, rape seed, soybean, pea and *Arabidopsis* (data not shown) suggest a wide applicability of the *codA* marker and reporter gene.

Experimental procedures

Nucleic acid manipulations

Standard methods described in Sambrook *et al.* (1989) were used for DNA and RNA manipulations. DNA-modifying enzymes were used according to the manufacturers instructions. The procedure of Dellaporta *et al.* (1983) with additional phenol, phenol/chloroform treatments, was used for extraction of DNA from plant tissue. Nucleic acids for hybridization were immobilized on Gene Screen, NEN DuPont. For Southern analyses conditions using dextran sulphate and formamide were used as outlined in method III of the Gene Screen instruction manual, except that 67°C and 0.015 M NaCl were used in the final wash. Northern analyses were according to the Gene Screen manual. Total RNA was extracted from 2–4 g of leaf material homogenized under liquid nitrogen and transferred frozen to a polypropylene centrifuge tube. To this 7.5 ml of 100 mM Tris pH 8.0, 50 mM EDTA, 500 mM NaCl, 5% mercaptoethanol, 4% Sarkosyl and 0.15 g ml^{-1} of CsCl were added. Extracts were shaken, incubated at 65°C for 15 min before being centrifuged at 10 000 r.p.m., 4°C, for 20 min. The supernatant was loaded on a 1.0 ml 5.7 M CsCl 100 mM EDTA cushion in SW 50 polyallomer tubes, and centrifuged at 160 000 × ***g*** for 17 h. Liquid above the cushion was removed and the tube above the cushion washed twice with water before removal of the cushion. Pelleted RNA was resuspended in 1.5 ml 10 mM Tris pH 7.5 and precipitated with 2.5 volume ethanol at −20°C for 30 min. After centrifugation at 10 000 r.p.m. for 20 min, RNA was dissolved in 1.5 ml 10 mM Tris, pH 7.5, 100 mM NaCl, and reprecipitated. RNA was finally dissolved in H_2O.

Construction of the codA *marker*

The *codA* coding sequence from position 1642 to the stop codon at position 2923 (Danielsen *et al.*, 1992) was PCR amplified using: (i) an N-terminal primer, 5′GAATCGATGTCGAATAACGCTTTACA AAC, changing the *E. coli* GTG translational start codon to ATG and introducing a *Cla*I site adjacent to the ATG; (ii) a C-terminal primer, 5′GATGATCAACGTTTGTAGTCGATGGCTTC, introducing a *Bcl*I site overlapping the TGA stop codon and eliminating the *Cla*I site with a point mutation at position 2913. Since the internal *Cla*I site is Dam methylated the *codA* coding sequence could then be subcloned as a *Cla*I/*Bcl*I fragment. The 35S promoter used in pNE7 extends from +4 to −1296, the 5′untranslated TMV Ω leader used was described by Scofield *et al.* (1992).

Plant growth

The *Lotus japonicus* 'Gifu' B-129 line was propagated and grown as described in Handberg and Stougaard (1992). The same conditions and nutrients were used for the tobacco plants. A medium consisting of half strength B5, without sucrose, and 0.4% gelrite was used for testing germination and growth. Pyrimidine bases, analogues, and orotate were dissolved at 10 mg ml^{-1} in 10 mM MES, pH 5.5. For negative selection a concentration of 5-FC at 250 μg ml^{-1} was used in standard experiments. Nucleosides and deoxynucleosides were dissolved at 100 mg ml^{-1} in 100 mM MES, pH 5.5. PALA was dissolved at 10 mg ml^{-1} in 30 mM MES, pH 5.5, 5-FOA at 10 mg ml^{-1} was adjusted to pH 6.0 with 1 M NaOH. When necessary, solutions were gently heated. All solutions were filter sterilized. Plate concentrations were: thymidine, cytidine, uridine, 500 μg ml^{-1}; deoxycytidine, 200 μg ml^{-1}; uracil, 500 and 100 μg ml^{-1}; cytosine, 500 and 50 μg ml^{-1}. Minimum inhibitory concentrations of 5-FC were: *Lotus*, 150 μg ml^{-1}; tobacco, 100 μg ml^{-1}.

Tissue culture

Transformation, regeneration and *in vitro* culture of *L. japonicus* was performed according to Handberg and Stougaard (1992). The leaf dish transformation procedure of Horsch *et al.* (1985) was used for tobacco. For negative selection 5-FC at 500 μg ml^{-1} was used as standard, the minimum inhibitory concentration was 400 μg ml^{-1}. Cytosine was used at concentrations up to 2 mg ml^{-1}, uracil up to 100 μg ml^{-1}.

Cytosine deaminase assay

Plant material was homogenized in 10-fold excess (v/w) of extraction buffer: 200 mM Tris-HCl pH 7.8, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, 2 mM PMSF. The standard assay was set up with 1 μCi 2-^{14}C cytosine in 10 μl of assay buffer, started by addition of 10 μl plant extract and then incubated at 37°C for 1 h. The assay buffer contained: 200 mM Tris-HCl pH 7.8, 1 mM DTT, 1 mM EDTA; 2 mM PMSF. For assays with excess cytosine the assay buffer contained additionally 10 mM cytosine. After 1 h a 5 μl or 10 μl assay mix was spotted on to a DC-Alufolien cellulose Merck 5552 TLC plate and dried using a blow dryer. The

TLC plate was developed in *n*-butanol/H_2O 86/14 v/v for 7 h. The TLC was then blow dried, covered with Saran wrap and autoradiographed. For quantitation of cytosine deaminase activity the spot containing 2-^{14}C uracil was counted in a scintillation counter. Protein in extracts was determined according to Spector (1978).

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